THE INFLUENCE OF GENE DOSAGE ON THE INHIBITION OF PROTEIN SYNTHESIS BY CRYPTOPLEURINE IN THE YEAST SACCHAROMYCES CEREVISIAE

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

INTRODUCTION

Cryptopleurine inhibits protein synthesis in the yeast Saccharomyces cerevisiae by prevention of amino acid incorporation into polypeptide, as studied in yeast polysomes (1, 8, 12, 22). Polyuridylic acid directed polyphenylalanine synthesis is inhibited by the drug in vitro (2, 4, 7, 8, 10, 22, 25). Resistance to cryptopleurine in yeast is due to a gene cryl that has been mapped in the range of 2.1 to 4.2 cM proximal to the mating type locus on chromosome III (7, 8, 16, 25). Skogerson, McLaughlin and Wakatama (25) showed that ribosomes from cryptopleurine resistant haploid mutant strains, carrying cryl, were more resistant to cryptopleurine in vitro than were ribosomes from sensitive haploid strains.

Meade, Riley and Manney (16) reported that diploid and higher ploidy mutant strains of yeast did not demonstrate normal Mendelian dominance-recessiveness relationships when assayed on agar plates containing a gradient of cryptopleurine concentrations. Instead of finding the mutant strains of the cryl locus to be recessive or dominant, they observed that strains exhibited various sensitivities to cryptopleurine which depended upon the ratio of cryl-7 resistant alleles to ploidy of the strain. Thus their homozygous sensitive diploid strains were most sensitive to cryptopleurine; homozygous resistant diploid strains were most resistant; and heterozygous diploid strains were of intermediate sensitivity. Unexpectedly, hemizygous diploid strains monosomic for chromosome III, and therefore possessing only one cryl allele, also were of intermediate sensitivity to cryptopleurine. Therefore, the expression of resistance to cryptopleurine seems to be independent of the sensitive

allele. When the ratios of cryptopleurine resistance alleles to ploidy equalled 1, the strains were most resistant, as measured by colony growth on gradient agar plates. Strains possessing lower ratios (cryl alleles/ploidy) displayed a proportionately lower resistance to cryptopleurine.

In order to investigate the effect of gene dosage on inhibition of protein synthesis by cryptopleurine in yeast, experiments were performed to measure the rates of protein synthesis in various cryptopleurine resistant strains. Tests were also done to determine the sensitivity to cryptopleurine in vitro of ribosomes from each strain.

Rates of protein synthesis were studied in diploid and monosomic diploid spheroplasts by measuring the incorporation of ^{3}H-leucine into acid precipitable peptide. Cell-free polyphenylalanine biosynthesis by ribosomes isolated from the various strains was measured by incorporation of ^{3}H-phenylalanine into acid precipitable polypeptide. These experiments determined the relative resistances to cryptopleurine of each strains' ribosomes and specifically determined if the monosomic diploid possessed ribosomes that were resistant to cryptopleurine in vitro. Radioactive protein synthesized in vivo and radioactive polyphenylalanine synthesized in vitro were examined by polyacrylamide gel electrophoresis to determine the sizes of polypeptide synthesized by each strain or its ribosomes in the presence of cryptopleurine. It was expected that these data, along with data that quantitate the rates of protein synthesis in vivo and in vitro would aid in designing a genetic model that further characterizes cryptopleurine resistance in yeast. A model is discussed that supports the collected data and indicates further experiments. This model suggests the existence of a gene not on chromosome III whose gene product interacts with the cryl gene product to effect the level of cryptopleurine resistance in yeast.

MATERIALS AND METHODS

Reagents. Deacylated E. coli. B transfer ribonucleic acid (tRNA), protein molecular weight markers, ^{3}H-phenylalanine (54 Ci/mM), and ^{3}H-leucine (48 Ci/mM) were obtained from Schwarz/Mann Laboratories. Polyuridylic acid (poly U), guanosine triphosphate (GTP), N,N,N',N'-tetramethylethylenediamine (TEMED), and sodium dodecyl sulfate (SDS) were purchased from Sigma. Adenosine triphosphate (ATP), acrylamide and N,N' methylene bisacrylamide were produced by Eastman. Dextran T-500 and Sephadex G-25 were obtained from Pharmacia Fine Chemicals Inc.. Cryptopleurine (CRY) was purchased from Chemsea Manufacturing Pty., Ltd., Peakhurst, New South Wales, Australia. Liquifluor and Protosol were obtained from New England Nuclear Corp.. Zymolyase was a product of Kirin Brewery Co., Ltd., Takasake Gumma Pref., Japan. Glusulase and insulin were obtained from Endo Laboratories and Mann Research Labs respectively. Components for media were purchased from Difco. Additional reagents were obtained at highest purity available from commercial sources.

Media. The culture media employed and their purposes are described below. All ingredients were added to distilled water and sterilized in the autoclave. Media were solidified with 20 g/l agar.

YEPD. Difco Yeast Extract, 10 g/l; Difco Bacto Peptone, 20 g/l; Dextrose, 20 g/l. YEPD is a rich complex medium used for routine culturing and stock maintenance (20).

YEPAD. YEPD with the addition of adenine at 80 mg/l, used for maintenance of adenine auxotrophs.

MV. Difco Yeast Nitrogen Base without Amino Acids and ammonium sulfate, 1.45 g/l; dextrose, 20 g/l; ammonium sulfate, 5.22 g/l. Minimal medium was used to score nutritional requirements (after Wickerham (28)).

SC. MV supplemented as follows: adenine, 20 mg/l; arginine, 20 mg/l; histidine, 10 mg/l; leucine, 60 mg/l; lysine, 40 mg/l; methionine, 20 mg/l; threonine, 350 mg/l; trytophan, 20 mg/l (stock solutions of tryptophan and threonine were filtered and the appropriate amounts added aseptically after autoclaving); uracil, 20 mg/l. This synthetic complete medium was used as a reference in the scoring of nutritional phenotypes.

-X. SC lacking one or more of the supplements listed above; e.g., SC-leu is SC minus leucine. Omission media were used for scoring nutritional phenotypes and as media for protein labeling with radioactive leucine.

CRY. YEPAD plates supplemented with cryptopleurine dissolved in 95% ethanol and added to the autoclaved medium at a final concentration of 5 µM. CRY was used to score resistance or sensitivity to cryptopleurine (16).

PET. Difco Yeast Extract, 10 g/l; Difco Bacto Peptone, 10 g/l; dextrose, 0.25 g/l; glycerol, 30 g/l. This medium was used to score for the respiratory deficient (petite) phenotype.

BTBgal. Difco Yeast Extract, 10 g/l; Difco Bacto Peptone, 20 g/l; galactose, 20 g/l; bromthymol blue (1% BTB in 95% ethanol), 33 g/l; 0.5 N NaOH (pH 8.0), 5 mg/l. This medium was used to score for the ability to ferment galactose. When galactose is fermented, metabolic

acids cause BTB to change from blue-green to yellow within 24 hours.

YEKAC. Difco Yeast Extract, 2.5 g/l; potassium acetate, 10 g/l. This was the sporulation medium (after McClary, Nulty and Miller (15)).

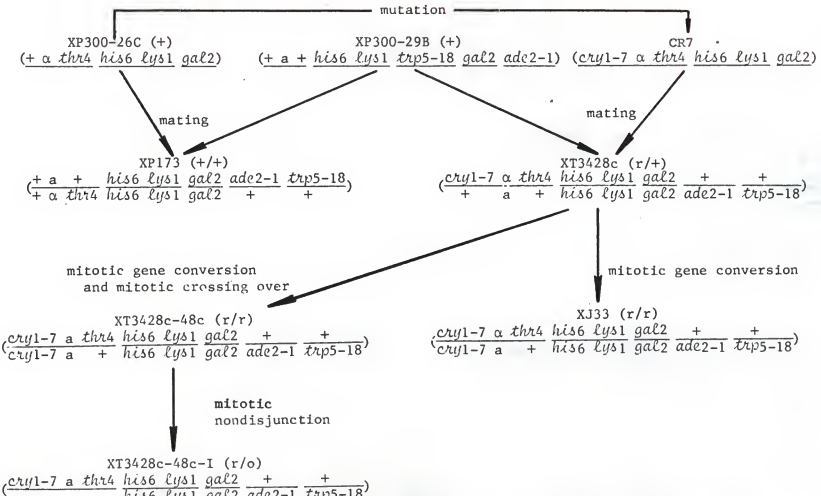
Incubation and growth. All incubations for growth and maintenance of strains were at 30°C. When liquid media were used, the flasks contained 20% of their nominal volume and were aerated by agitation on a rotary platform shaker at 200 rpm. Yeast cells prepared for spheroplasting were grown in SC-leu medium and harvested at approximately 1 x 10^7 cells/ml. Yeast cells for preparation of ribosomes and S100 were grown in YEPD to a density of 2.5 x 10^7 cells/ml, harvested by centrifugation at 10,000 x g and stored at -20°C until used.

Yeast strains and nomenclature. The isolation of cryptopleurine resistant yeast mutants and construction of diploid and monosomic diploid strains used in these experiments have been described by Meade, Riley and Manney (16), and Riley and Manney (19). These strains and their genotypes are summarized in Table 1. The genetic marker symbols are those given by Plischke, VanBorstel, Mortimer and Chom (18) and are listed in Appendix I. Those genes grouped by the underline reside in the order given on the same linkage group (24). For ease in designating the cryl genotype of the strains, r will be used for the cryptopleurine resistant allele, + for the wild-type sensitive allele, and o to designate the missing chromosome III in aneuploid strains. The common genetic background of these strains is indicated in Figure 1. The parent haploid strains are XP300-26C (+) of mating type α and XP300-29B (+) of mating type a. The cryptopleurine resistant haploid strain, CR7 (r), is a mutant isolated from XP300-26C (+). Haploid yeast strains of opposite

Table 1. Strain list

Strain		Genotype
XP300-29B	+	+ MATa + *his6 lys1 gal2 ade2-1 trp5-18*
XP300-26C	+	+ MATα *thr4 his6 lys1 gal2*
CR7	r	*cry1-7* MATα *thr4 his6 lys1 gal2*
XP173	+/+	+ MATa + *his6 lys1 gal2 ade2-1 trp5-18* + MATα *thr4 his6 lys1 gal2* + +
XT3428c	r/+	*cry1-7* MATα *thr4 his6 lys1 gal2* + + + MATa + *his6 lys1 gal2 ade2-1 trp5-18*
XT3428c-48c	r/r	*cry1-7* MATa *thr4 his6 lys1 gal2* + + *cry1-7* MATa + *his6 lys1 gal2 ade2-1 trp5-18*
XT3428c-48c-I	r/o	*cry1-7* MATa *thr4 his6 lys1 gal2* + + *his6 lys1 gal2 ade2-1 trp5-18*
XJ33	r/r	*cry1-7* MATα *thr4 his6 lys1 gal2* + + *cry1-7* MATa + *his6 lys1 gal2 ade2-1 trp5-18*

Figure 1. Geneology of strains used in these studies, showing common genetic background.


mutation
XP300-26C (+)
(+ α thr4 his6 lys1 gal2)
XP300-29B (+)
(+ a + his6 lys1 trp5-18 gal2 ade2-1)
CR7
(cry1-7 α thr4 his6 lys1 gal2)
mating
mating
XP173 (+/+)
XT3428c (r/+)
mitotic gene conversion
and mitotic crossing over
mitotic gene conversion
XT3428c-48c (r/r)
XJ33 (r/r)
mitotic
nondisjunction
XT3428c-48c-I (r/o)

mating type can mate to form diploid strains. The mating of XP300-26C (+) and XP300-29B (+) yielded the homozygous sensitive strain XP173 (+/+); mating of XP300-29B (+) and CR7 (r) yielded the heterozygous strain XT3428C (r/+). The homozygous resistant strain XJ33 (r/r) was derived from the heterozygous diploid strain by mitotic gene conversion at the cryl locus. Another homozygous resistant strain XT3428C-48C (r/r) was derived from the heterozygous strain, presumably through mitotic crossing over at the mating type locus and mitotic gene conversion at the cryl locus. The monosomic diploid XT3428C-48C-I (r/o) was derived from the homozygous resistant strain XT3428C-48C (r/r) through loss of chromosome III.

The tester strains used to confirm the mutant strains' genotypes were derived from strains obtained from R.K. Mortimer (University of California, Berkeley). These strains and their genotypes are listed in Table 2. The two strains, XT1219-1A and XT1219-18A were used to determine the mating types of the spores. These test strains were deficient for galactose fermentation at the gal1 locus and the spores that were tested were deficient at the gal2 locus. When a haploid spore was cross-streaked with each of these strains, it was able to mate with the strain of opposite mating type and the galactose fermentation deficiencies of each strain were complemented by the other strain. Thus, the diploid progeny of the mating were able to ferment galactose changing the BTB medium to yellow. When a haploid spore mated with XT1219-1A, of mating type a, then the haploid spore was of mating type α.

The strains X1069-2D and S1795A were used to determine nutritional deficiencies in diploid spores. These test strains carry the histidine auxotrophy at the his4 locus while the spores that were tested carried

Table 2. Tester strain list

Strain	Genotype
X1069-2D-CR	*his4 leu2 cry1-7* MATα *thr4 trp5 leu1 ade6 ura1 ade1 met2* + + +
XT1172-22C	+ + + MATa + *trp5 leu1 ade6* + + + + + +
XT1219-1A	+ + + MATa + + + + + *ade1* + *gal1 trp1 his2*
XT1219-18A	+ + + MATα + + + + + *ade1* + *gal1 trp1 his2*
X1069-2D	*his4 leu2* + MATα *thr4 trp5* + + *ura1 ade1 met2* + + +
S1795A	*his4* + + MATa + *trp5* + *ade6 ura1* + + + + +
XP1069	*his4 leu2 cry1-7* MATα *thr4 trp5* + + *ura1 ade1 met2* + + +
	+ + *cry1-7* MATα *thr4 trp5 leu1 ade6* + + + + + +

the histidine auxotrophy at the his6 locus. The diploid spores were cross-streaked with each of these strains and if the spores were able to mate (i.e., not of mating type a/α) they mated with the test strain of opposite mating type. When each strains' histidine requirements were complemented, the progeny of the mating were able to grow on SC-his medium.

Since the r/o strain possessed only one mating type locus on chromosome III, it could not sporulate to form a tetrad. A cryptopleurine resistant diploid strain was isolated to mate with the r/o strain and form a tetraploid strain that could sporulate. The cryptopleurine resistant strain X1069-2D-CR was isolated from the sensitive strain X1069-2D. The strain subsequently was crossed with the strain XT1172-22C to form a strain heterozygous for both mating types (a/α) and cryptopleurine resistance (r/+). Mitotic crossing over between the centromere and the cryl-7 locus yielded XP1069, a strain homozygous for cryptopleurine resistance and the α mating type. XP1069 was crossed with the r/o strain to form a tetraploid strain that was sporulated and used to confirm the genotype of the r/o strain.

Genetic Analysis. The genotypes of the three normal diploid strains (r/r, r/+ and +/+) were confirmed by tetrad analysis. The strains were grown for 24 hours on YEPAD plates for presporulation growth, transferred to YEKAC plates and incubated 3 to 4 days. Sporulation occurred and asci were dissected by the method of Johnston and Mortimer (12). The sporulation mixture was treated with a 1:20 dilution of glusulase for 12 minutes and a small amount of the mixture was spread along the edge of a thin YEPAD agar slab with a loop. The four spores

from each of the asci were isolated by micromanipulation, incubated for 2 to 4 days on YEPAD plates, and transferred to YEPAD master plates. The master plates containing individual spores were scored for nutritional phenotype by replica plating to appropriate media. As a test for mating type, each spore was cross-streaked with the tester strains XT1219-1A and XT1219-18A, incubated for 24 hours, and replica plated to MV or BTBgal agar plates. Each of the disomic diploid strains produced tetrads with the expected nutritional segregation frequencies.

The genotype of the monosomic diploid (r/o) was verified by mating it with the diploid strain XP1069 and isolating a zygote diploid which was then sporulated and dissected giving diploid spores. The spores were tested for nutritional phenotype by replica plating. They were cross streaked with XT1219-1A and XT1219-18A to determine mating type. Since these were diploid spores, heterozygous spores (a/α) were non-maters. Each of the spores was also cross streaked with the histidine auxotrophs X1069-2D and S1795A to determine nutritional phenotypes. The spores were scored for sporulation on YEKAC agar plates by microscopic examination to determine whether nonmating spores were sporulators. The results of sporulation tests in conjunction with the mating type tests and thr4 segregation frequencies were used to eliminate false asci. Only data from tetrads with four germinated spores were analyzed. All the isolated diploid spores showed the expected ratios of nutritional phenotypes, mating types and sporulation.

Preparation of spheroplasts. The spheroplast technique used was a modification by T.R. Manney and R. Corfman (personal communication) of the technique used by Hartwell (9). The cells were harvested by centri-

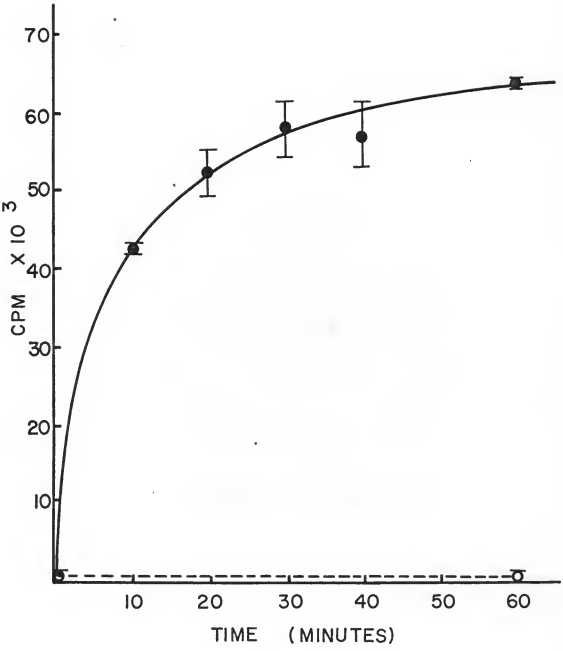
fugation at 1,500 x g for 5 minutes. They were resuspended in deionized-distilled water and centrifuged as before in a graduated conical centrifuge tube. The volume of cells was converted to weight of cells (there are approximately 0.98 grams yeast cells per milliliter of packed cells). The cells were suspended in an osmotically supportive buffer solution containing 0.6 M mannitol and 0.01 M potassium phosphate (pH 7.0). Approximately 5 mls buffer per 0.1 gms cells was enough to suspend the cells. The cell number was measured by counting samples with a Coulter Counter (Model Zf, 100 µm aperature) after sonication for 10 seconds and dilution in 0.85% NaCl. The 5 ml samples of suspended cells were mixed with 0.1 ml 2-mercaptoethanol and zymolyase stock solution was added at a level of 50 mg enzyme per gram cells. The zymolase stock solution was prepared by dissolving the enzyme at 10 mg/ml in 0.1 M potassium phosphate buffer (pH 7.0) and 0.6 M mannitol. The reaction mixtures were incubated at 22°C on a rotary platform shaker at 200 rpm for one hour. Spheroplasts were centrifuged at 1,500 x g for 5 minutes, washed in the same buffer (0.6 M mannitol and 0.01 M potassium phosphate (pH 7.0)) and recentrifuged. The spheroplasts then were suspended in SC-leu containing 0.6 M mannitol and 0.01 potassium phosphate (pH 7.0) at 5 x 10^7 spheroplasts/ml. Untreated cells and spheroplasts were diluted in 0.85% NaCl and plated on YEPAD at 250 cells per plate and 500 cells per plate respectively. Plating efficiency was 75% for cells and 0.02% for spheroplasts. Therefore, approximately 99.98% of the yeast were rendered spheroplasts by this procedure.

Protein synthesis in spheroplasts. The time required for spheroplasts to resume protein synthesis after treatment with mercaptoethanol

and zymolyase was determined by incubating spheroplasts from the sensitive diploid for various times at 30°C followed by a 10 minute pulse of ^{3}H-leucine (48 Ci/mM) to determine the rate of protein synthesis. Reactions of 1 ml contained 5 x 10^{7} spheroplasts in growth media (SC-leu plus 0.6 M mannitol and 0.01 M potassium phosphate (pH 7.0)). After incubation in a shaker bath at 30°C for the times indicated, 1 µCi ^{3}H-Leu was added to duplicate 1 ml samples. After an additional 10 minutes, the reactions were terminated by addition of SDS (1% w/v) and trichloroacetic acid (10% w/v), they were heated at 90°C for 15 minutes and then incubated in an ice water bath for 15 minutes. Acid precipitable proteins were collected on glass fiber filters, and radioactive proteins were assayed in a liquid scintillation counter. The rate of protein synthesis in spheroplasts increased rapidly during the initial 10 minutes after return to 30°C and reached a plateau by approximately 30 minutes as shown in Figure 2. Protein synthesis depended upon incubation at 30°C, since control samples that were retained at 4°C for up to 60 minutes did not incorporate radioactivity (open circles in Figure 2). Based upon this experiment, 30 minutes at 30°C was chosen for a standard preincubation of spheroplasts in further experiments.

In order to determine the time period necessary for cryptopleurine to inhibit protein synthesis, the drug was added in the range of 1 x 10^{-5} to 1 x 10^{-7} M to spheroplast reaction samples as before. One set of samples was incubated for 15 minutes, inhibited with the drug for 15 minutes and pulse labeled with 1 µCi ^{3}H-leucine for 10 minutes. The other set of samples was incubated for 25 minutes, inhibited by the drug for 5 minutes and pulse labeled with 1 µCi ^{3}H-leucine for 10

Figure 2. The rates of ^{3}H-leucine incorporation into protein in yeast spheroplasts. Spheroplasts of the +/+ strain were incubated at 30°C for the time periods indicated and then were pulse labeled for 10 min. with ^{3}H-Leu. Trichloroacetic acid precipitated radioactivity was measured in spheroplast lysates. Symbols: (●) incubation and pulse labeling at 30°C and (○) incubation and pulse labeling at 0°C.


70
60
50
40
30
20
10
CPM X 10 3
10
20
30
40
50
60
TIME (MINUTES)

minutes. The reaction samples were terminated and assayed as previously described. The two drug incubation time periods produced equivalent inhibitions of spheroplast protein synthesis. A 5 minute incubation time with cryptopleurine was chosen for further experiments since the drug sufficiently inhibited spheroplast protein synthesis in that time period.

The effects of cryptopleurine on protein synthesis were determined in reactions that contained spheroplasts from various strains in 1 ml of the same growth media. After incubation at 30°C for 25 minutes, cryptopleurine was added to the samples at concentrations from 1×10^{-7} to 1×10^{-5} M for 5 minutes. The samples were then pulse labeled for 10 minutes with 1 µCi of ^{3}H-Leu and were processed as described above.

Reaction mixtures analyzed by gel electrophoresis were identical to the above samples, except that they were pulse labeled with 5 µCi/ml ^{3}H-Leu for 20 minutes at 30°C. After chilling the mixtures on ice, the spheroplasts were harvested by centrifugation, lysed in 0.1 M NaOH, incubated at 37°C for 30 minutes and neutralized with HCl.

Preparation of ^{3}H-Phe-tRNA. The optimal conditions for aminoacylating tRNA were (in 15 ml): 0.3 M Tris hydrochloride (pH 8.1); 0.01 M KCl; 0.02 M magnesium acetate; 0.01 M ATP; 1635 A_{260} units tRNA; 25 mg dialyzed dextran phase yeast protein (25); and 2.92 mCi of ^{3}H-Phe. The ^{3}H-Phe was prepared in a 40 ml conical tube and all reagents except the dialyzed protein were added and mixed at 37°C for 5 minutes for equilibration. The reaction was initiated by addition of the dialyzed protein fraction and incubated at 37°C for 30 minutes. One tenth volume of 1.0 N sodium acetate (pH 5.0) was added on ice to the solution to

stabilize the aminoacyl-tRNA. The solution was mixed gently with an equal volume of phenol on ice for 10 minutes and centrifuged at 1,500 x g for 5 minutes. The aminoacylated tRNA was removed in the upper aqueous phase, and the remaining phenol phase was reextracted with buffer. The pooled aqueous phases were chromatographed on a G-25 medium column in 1.0 mM sodium acetate (pH 5.0) to remove free ^{3}H-Phe. From each column fraction 20 µl was precipitated with 10 ml of 5% trichloroacetic acid (TCA) and 5 ml of 0.01 N HCl to determine the amount of acid precipitable ^{3}H-Phe-tRNA. Radioactivity was determined by using Liquifluor scintillation fluid in a scintillation counter at 22% efficiency. The fractions with ^{3}H-Phe-tRNA were pooled and lyophilyzed. The lyophilyzed ^{3}H-Phe-tRNA samples were stored desicated at -20°C. The efficiency of amino-acylation was generally 0.4 to 0.5% (pmole ^{3}H-Phe/pmole tRNA) in these preparations.

Preparation of ribosomes and elongation factors. Elongation factors (S100) and ribosomes were prepared by the dextran-polyethylene glycol two phase extraction procedure of Skogerson, McLaughlin and Wakatama (25).

Development of polyphenylalanine synthesis assay. Cryptopleurine is insoluable in water and thus was dissolved at a concentration of 0.02 M in 95% ethanol. To solubilize the drug, acetic acid was added also to 0.02 M. Then the solution was diluted in water to 1 x 10^{-3} M and neutralized with NaOH. Cryptopleurine concentrations were measured using the extinction coefficient of 5.75 x 10^{4} l/cm·mole, at 258 nm (3). Since there was residual ethanol and acetic acid in dilute cryptopleurine solutions, the same concentration of ethanol and acetic acid always were used in control experiments. The rate of polyphenylalanine synthesis in reactions containing this solvent alone (i.e., acetic acid

and ethanol) was unaffected by solvent to a concentration of 1×10^{-4} M acetic acid (see Figure 3). At a concentration of 5×10^{-4} M the solvent itself inhibited polyphenylalanine synthesis by over 90%. This assay was therefore restricted to solvent or cryptopleurine concentrations of 1×10^{-4} M or less. The percent ribosome activities for reactions that contained cryptopleurine were computed by the formula:

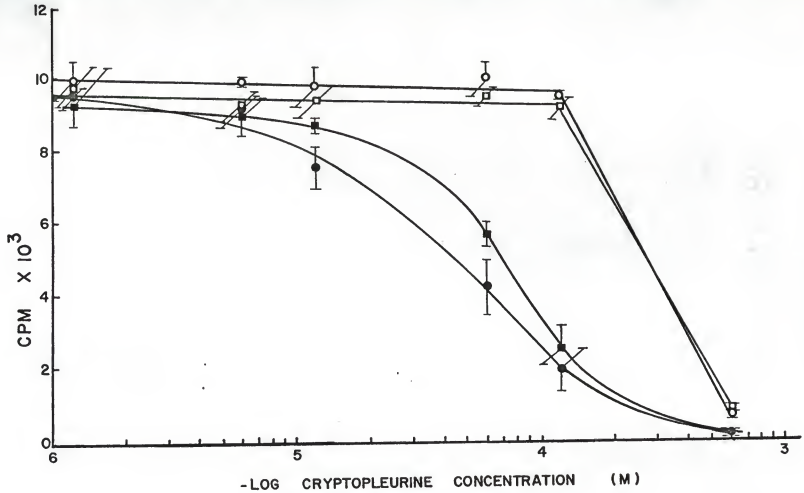
$$\left(1 - \frac{\text{control cpm} - \text{cryptopleurine cpm}}{\text{control cpm}}\right) \times 100.$$

Confidence limits in experimental data were determined by the Student T-test and by use of the standard deviation.

Polyphenylalanine synthesis. Reaction mixtures contained in 0.1 ml: 0.05 M Tris acetate (pH 7.0); 0.05 M NH_4Cl; 0.02 M dithiothreitol (DTT); 0.08 M $MgCl_2$; 0.494 A_{260} units of poly U; 0.001 M GTP; 33 µg of dialyzed S100 protein from the (+/+) strain; 0.2 A_{260} units of ribosomes; 6 pmole of ^{3}H-Phe-tRNA; and the indicated concentration of cryptopleurine. These conditions gave optimal concentrations for all reagents except for the amount of ribosomes which was limiting. After incubation for 15 minutes at 37°C, reactions were terminated by the addition of 0.5 ml of 10% TCA, heated at 90°C for 15 minutes and quickly cooled by addition of 1 ml cold 10% TCA. After 15 minutes on ice, the samples were filtered and assayed as described above.

Reaction mixtures analyzed by polyacrylamide gel electrophoresis were as above but their volumes were five times larger (i.e., 0.5 ml). After an incubation for one hour at 37°C, these reactions were terminated by the addition of NaOH to a final concentration of 0.1 M and incubated at 37°C for an additional 30 minutes. The samples were neutralized with 1 M HCl and lyophilyzed.

Figure 3. Inhibition of polyphenylalanine synthesis by cryptopleurine in solvent and by solvent (ethanol + acetic acid) alone. Cryptopleurine inhibited ribosomes from (●) +/+ and from (■) r/r. Solvent inhibited ribosomes from (○) +/+ and from (□) r/r. All points were determined by duplicate reaction mixtures and the error bars indicated are two standard deviations.


12
10
8
6
4
2
0
CPM X 10³
6
5
4
3
-LOG CRYPTOPLEURINE CONCENTRATION (M)

Polyacrylamide gel electrophoresis. The molecular weights of the protein products synthesized by spheroplasts and of ^{3}H-polyphenylalanine whose synthesis in vitro was directed by poly U were analyzed by Laemmli's method of sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (14). The apparatus was assembled and the bottom of gel tubes or slabs were covered with Parafilm to support the gels during polymerization. Gels containing 4 or 5% (stacking gels) and 15 or 20% (resolving gels) acrylamide were prepared from a stock solution of 30% by weight and 0.8% by weight of N,N' bis-methylene acrylamide. Buffers in the resolving gel were 0.375 M Tris hydrochloride (pH 8.8) and 0.1% SDS. The above mixture was degassed under vacuum for 3 minutes and polymerized by the addition of 0.025% by volume of tetramethylethylenediamine (TEMED) and ammonium persulfate. The mixture was quickly poured into glass tubes for 10 cm tube gels or between vertical glass plates to form a 10 cm slab gel and were overlaid gently with water to polymerize for one hour. The 4 or 5% stacking gels contained 0.125 M Tris hydrochloride (pH 6.8) and 0.1% SDS and were polymerized by the same method as for the resolving gel. The water was removed from the top of the resolving gel and the stacking gel was poured over the resolving gel to a depth of 1 cm and allowed to polymerize for 30 minutes to one hour.

Protein molecular weight (MW) standards used to calibrate the gels were bovine serium albumin (BSA), MW 65,400 daltons; ovalbumin (OV), MW 45,000 daltons; chymotrypsinogen (CHY), MW 25,000 daltons; cytochrome C (CYT C), MW 13,400 daltons; insulin (I), 5,700 daltons. The molecular weight standards were dissolved in a dye solution containing 0.057 M Tris hydrochloride (pH 6.8); 0.001% bromphenol blue (BPB); 1.83% SDS.;

10.83% glycerol; 1.33 M urea; 0.017% acetic acid; 0.017% basic fuscin; and 0.917% 2-mercaptoethanol. Each protein standard was added at a final concentration of 4 to 5 µg per gel.

The gels were placed in the electrophoresis chamber filled with the electrode buffer (pH 8.3) containing 0.025 M Tris, 0.192 M glycine; and 0.1% SDS. Electrophoresis samples were dissolved in a small volume of electrophoresis buffer containing 0.0625 M Tris hydrochloride (pH 6.8); 1 M 2-mercaptoethanol; 0.001% bromphenol blue; 2% SDS; and 10% glycerol and heated in a boiling water bath for three minutes. Samples were loaded into the gel sample wells, and electrophoresis was carried out with a current of 5 mA per gel (tube gels) or 40 mA (slab gels) until the BPB marker was 0.5 cm from the bottom of the gel (about five hours). The gels were removed from the glass using water pressure from a 10 ml syringe. The gels containing molecular weight standards were stained overnight in a 0.2% Coomassie brillant blue, 50% methanol and 10% TCA. They were destained for 5 hours at 37°C in 50% methanol and 10% TCA and diffusion-destained by repeated washings in 10% methanol and 10% TCA. When protein bands were visible, the positions of bands and the total gel length were recorded. The remaining gels were frozen, fractionated into 2 mm slices, and placed into Liquifluor scintillation fluid containing 3% Protosol. The sample slices were heated for 12 hours at 37°C to promote release of proteins from the gel. Yields of radioactivity were analyzed in a LS-100C Beckman scintillation counter at 22% efficiency.

RESULTS

Cryptopleurine resistance in spheroplasts. Meade, Riley and Manney (16) observed that yeast strains possessing different ratios of cryptopleurine-resistance alleles to ploidy demonstrated different sensitivities to cryptopleurine on agar plates containing a gradient of cryptopleurine concentrations. They found that resistance to the drug increased in proportion to that ratio, and that the monosomic diploid strain (r/o) hemizygous for the resistant allele was as sensitive to the drug as was the heterozygous strain (r/+). To confirm that cryptopleurine inhibits protein synthesis and to better quantitate resistance of the various strains to the drug, ratios of protein synthesis were analyzed in spheroplasts treated with cryptopleurine.

Spheroplasts prepared from several yeast strains, both sensitive and resistant to cryptopleurine, were used to determine the effect of cryptopleurine on their rates of protein synthesis as described in Methods. The effects of cryptopleurine on several strains are shown in Figure 4. Rates of protein synthesis in the presence of 5×10^{-7} M cryptopleurine were also measured in six identical samples of spheroplasts from each diploid yeast strain to verify their relative sensitivities to the drug. The results are presented in Table 3.

From Figure 4 one observes that the r/r diploid and r haploid strains were more resistant than the diploid r/+ and monosomic diploid r/o strains. The r/+ and r/o strains were more resistant than the sensitive diploid +/+ and haploid + strains. The concentrations of cryptopleurine required for 50% inhibition of protein synthesis were

Figure 4. Inhibition of spheroplast protein synthesis by cryptopleurine. Details of this assay are given in the text. Data points at 5×10^{-7} M cryptopleurine have 7 d.f. while all other data was derived from duplicate reactions. Symbols: ● +/+, ○ +, ● r/o, ▲ r/+, □ r, ■ r/r.

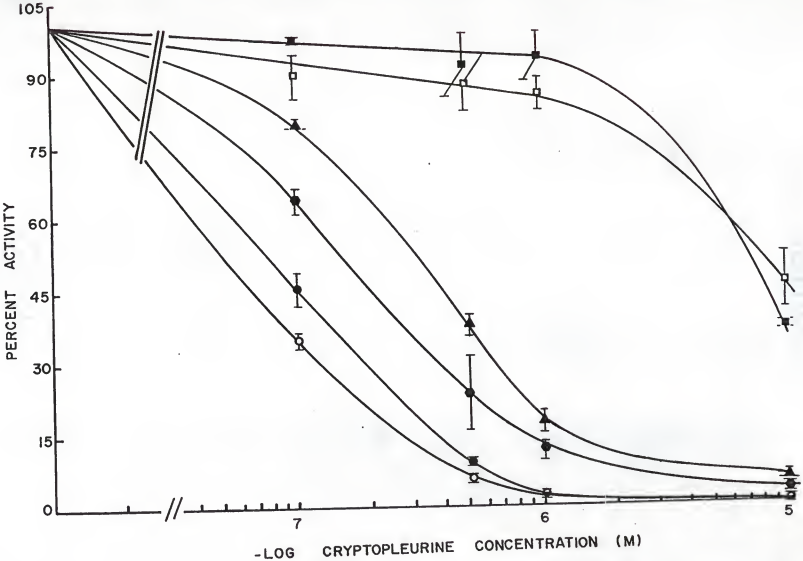

105
90
75
60
45
30
15
0
PERCENT ACTIVITY
7
6
5
-LOG CRYPTOPLEURINE CONCENTRATION (M)

Table 3. Sensitivity of yeast spheroplasts to cryptopleruine (5×10^{-7} M)

	^{3}H-leucine incorporated cpm/10 min. pulse		
Genotype	Solvent along	+cryptopleurine	Activity remaining %
r/r	64376 ± 4610*	58666 ± 5564*	91.1 ± 8.5*
r/+	49685 + 7528	19119 ± 915	38.5 ± 1.8
r/o	59446 ± 5006	17134 ± 656	28.8 ± 1.0
+/+	71305 ± 6805	6659 ± 180	9.3 ± 0.0

*95% confidence limits based upon the t-test using 5 d.f.

approximately forty-fold higher in the r/r and r resistant strains than in the r/+ and r/o strains. The concentrations of cryptopleurine required for the equivalent inhibitions were approximately three fold higher for the r/+ and r/o than for the +/+ and + strains. These data are summarized in Table 4 where the ratios of cryptopleurine resistant alleles to ploidy also are indicated. The results of the replicate tests that contained 5×10^{-7} M cryptopleurine as tabulated in Table 3 are comparable to the data presented in Figure 4 and indicate the same order of resistance by the various strains to cryptopleurine. These results agree with the relative sensitivities for growth observed by Meade, Riley and Manney on gradient agar plates (16). Strains possessing a ratio of 1 (r/r and r) were most resistant, strains with a ratio of ½ (r/+ and r/o) were of intermediate resistance and strains with a ratio of 0 were most sensitive.

The size of proteins synthesized by spheroplasts in the presence of cryptopleurine. Cryptopleurine inhibits elongation of polyphenylalanine peptide (25), and one might expect that ribosomes from resistant strains would allow elongation to procede while ribosomes from sensitive strains would block progression of both resistant and sensitive ribosomes during their progression along the mRNA. Thus one expectation was that the yeast strain possessing only resistant ribosomes (r/r and r/o) would synthesize longer proteins in the presence of cryptopleurine while the heterozygous diploid (r/+) would synthesize only shorter polypeptides.

The sizes of proteins synthesized in spheroplasts from the four diploid strains in the presence of cryptopleurine was investigated by gel electrophoresis. The concentration of cryptopleurine was 1×10^{-6} M,

Table 4. Inhibition of protein biosynthesis in yeast spheroplasts.

Yeast strain genotype	*cry1-7* alleles/ploidy	Cryptopleurine conc. ID_{50} μM*
r/r	2:2= 1	9.2
r	1:1= 1	7.8
r/+	1:2= ½	0.28
r/o	1:2= ½	0.165
+/+	0:2= 0	0.075
+	0:2= 0	0.054

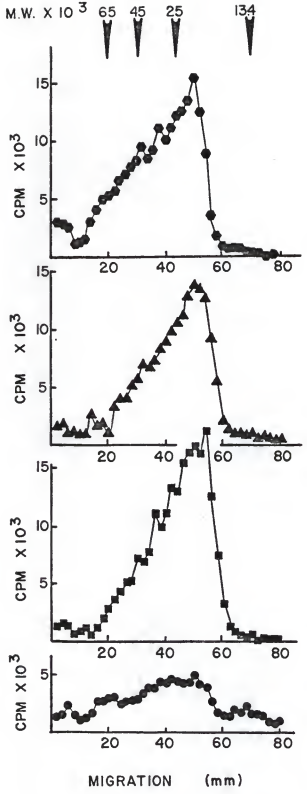
* Inhibitory Dose that causes 50% inhibition of the rate of protein biosynthesis.

since that concentration best distinguished between resistant and sensitive strains (see Figure 4). The electrophoretic patterns of proteins labeled with ^{3}H-Leu are illustrated in Figure 5. Migration of several standard proteins also is indicated at the top of the figure.

Spheroplasts from each diploid strain synthesized ^{3}H-labeled proteins that were hetergeneous in size. The proteins varied in size between 10,000 and 65,000 daltons with a molecular weight peak in the range of 21,000 daltons. As is apparent in Figure 5, spheroplasts from the three diploid strains possessing at least one r allele (r/r, r/o and r/+) all synthesized proteins that could not be distinguished on the basis of molecular weight. Spheroplasts from the three strains possessing r alleles also synthesized more protein than did the sensitive diploid strain (+/+). Since the r/+ spheroplasts synthesized proteins of the same size and amount as the strains containing only r alleles, the results of these experiments suggest that sensitive ribosomes are not blocking the progression of resistant ribosomes along mRNAs. Therefore, if sensitive ribosomes are formed, it appears that cryptopleurine may inhibit the binding of sensitive ribosomes to polysomes as well as their progression along the message.

Ribosomes and S100 yields. Several investigators (2, 4, 7, 8, 10, 22, 25) have reported the ribosome to be the site of cryptopleurine resistance in haploids. If resistance to cryptopleurine depends on the number of resistant ribosomes per cell then one might expect the r/+ and r/o strains to possess equal resistance to the drug if ribosome concentrations are regulated by a locus on chromosome III. The diploid resistant strain (r/r) would possess more resistant ribosomes than the

Figure 5. Electrophoresis of ^{3}H-proteins synthesized by spheroplasts. The samples were prepared and analyzed by polyacrylamide gel electrophoresis as described in the text. The electrophoretic mobility of the standard proteins are indicated at the top: bovine serum albumin (BSA), 65,000 daltons; ovalbumin (OV), 45,000 daltons; chymotrypsinogen (CHY), 25,000 daltons; cytochrome C (CYT C), 13,400 daltons. The total number of ^{3}H-cpm incorporated by the spheroplast samples were for +/+, 1.06×10^{5} (●); for r/r, 2.23×10^{5} (■); for r/+ 1.83×10^{5} (▲); and for r/o, 2.15×10^{5} (◆).


M.W. X 10 3
65
45
25
134
CPM X 10 3
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5
20
40
60
80
MIGRATION (mm)

r/+ and r/o strains and thus would be more resistant to cryptopleurine as was shown in spheroplasts (see Figure 4). Yields of ribosomes and S100's were analyzed to determine if strain differences in yield among the strains might correlate with the patterns of resistances to cryptopleurine.

Ribosomes and elongation factors (S100) were prepared from the four diploid strains as described above. The yields of these components from each strain are summarized in Table 5. From the data in Table 5, it is apparent that the monosomic diploid strain (r/o) yielded somewhat fewer ribosomes than did the sensitive strain (+/+) and resistant strain (r/r), but only slightly less than did the heterozygous strain (r/+). Yields of S100 proteins from all the strains were comparable. The r/o strain produced approximately 76% as many ribosomes as did the r/r strain and 87% as many ribosomes as did the r/+ strain. If the resistance to cryptopleurine is proportional to the number of resistant ribosomes per cell, then the r/o strain should be 76% as resistant as the r/r strain and more resistant than the r/+, which has only a portion of its ribosomes that are resistant. Thus differences in ribosome yield and thus resistant ribosome content do not account for the r/o strain's lower sensitivity to cryptopleurine.

Cryptopleurine resistance of ribosomes in polyphenylalanine synthesis. Skogerson, McLaughlin and Wakatama (25) found the ribosome to be the site of cryptopleurine resistance in mutant haploid strains. Others (7, 22) observed that ribosomes from heterozygous diploid strains (r/+) possessed intermediate resistances to cryptopleurine.

^{3}H-polyphenylalanine synthesis was assayed with ribosomes prepared

Table 5. Ribosome and elongation factor (S100) yields

Strain / Preparation data	+/+		r/r		r/+		r/o	
	Ribosomes	S100	Ribosomes	S100	Ribosomes	S100	Ribosomes	S100
Total A_{260}	1986.5	367.5	1543.0	318.3	1540.0	382.0	1185.0	247.5
Total A_{280}	1025.3	277.3	1004.4	241.4	808.5	279.0	695.2	212.5
A_{260}/gm of cells*	102.9	19.0	93.9	19.4	81.7	20.3	71.0	14.8
A_{280}/gm of cells*	53.1	14.4	61.6	14.7	42.9	14.8	41.6	12.7
mg ribosomes/gm of cells[a]	8.6	---	7.8	---	6.8	---	5.9	---
mg S100/gm of cells[b]	---	7.9	---	8.0	---	7.5	---	8.4

* wet weight of cells for these preparations were: +/+, 19.3 gm; r/r, 16.4 gm; r/+, 18.8 gm; r/o, 16.7 gm.

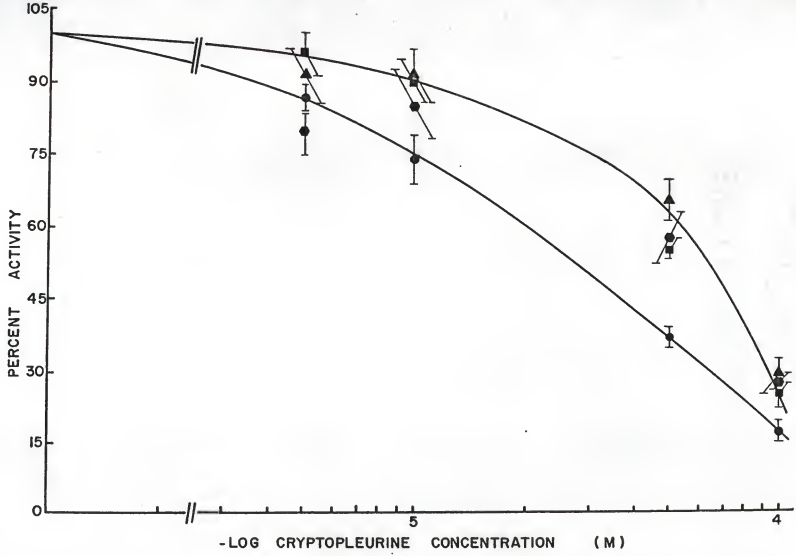
[a] based upon 12 A_{260}/mg ribosomes (21).

[b] based upon (protein) mg=1.55 A_{280}-0.76 A_{260} (23).

from each of the four diploid strains to determine if ribosomes from these strains showed similar sensitivities to cryptopleurine or if the ribosomes' sensitivities reflected the properties observed in spheroplasts. ^{3}H-polyphenylalanine synthesis was assayed as described above using elongation factors (S100) from the sensitive strain (+/+). A comparison of the effects of cryptopleurine on polyphenylalanine synthesis using ribosomes from the four diploid strains is presented in Figure 6. The cryptopleurine concentrations necessary to inhibit ^{3}H-polyphenylalanine synthesis in vitro were considerably higher than those necessary to inhibit protein synthesis in living spheroplasts. Ribosomes from the strains that possessed at least one resistant allele (r/r, r/o and r/+) were indistinguishable from each other by this assay and were more resistant to cryptopleurine than were ribosomes from the sensitive strain (+/+). The concentration of cryptopleurine required for 50% inhibition of polyphenylalanine synthesis was approximately 2x higher for all the strains that contained at least one resistant allele than for the diploid sensitive strain (+/+). Thus strains that possessed at least one r allele produced ribosomes that were more resistant to cryptopleurine in vitro.

To determine if ribosomes from the resistant strain tested were causing the same amount of ^{3}H-Phe incorporation into ^{3}H-polyphenylalanine in the presence of cryptopleurine as in the absence of the drug, yields of polyphenylalanine produced per ribosome (^{3}H-Phe pmoles per A_{260} of ribosomes) were computed from the data pf Figure 6 at a cryptopleurine concentration of 5×10^{-5} M (see Table 6). Each of the strains incorporated equivalent amounts of ^{3}H-Phe into polyphenylalanine in the

Figure 6. Inhibition of polyphenylalanine synthesis by cryptopleurine. Ribosomes were prepared from: +/+ (●), r/r (■), r/+ (▲), and r/o (●). Polyphenylalanine synthesis was carried out in 8 replicate reactions for 15 min. in the presence of various concentrations of cryptopleurine as described in the text. Only elongation factors from the sensitive strain, +/+, were used. The error bars indicate 90% confidence limits on the data as calculated from the t-test, d.f.=7.


105
90
75
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45
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15
0
PERCENT ACTIVITY
5
4
-LOG CRYPTOPLEURINE CONCENTRATION (M)

Table 6. Polyphenylalanine produced per ribosome in polyphenylalanine synthesis*

	(pmole ^{3}H-phe/A_{260} ribosomes/15 min.)	
Strain	+ cryptopleurine 5 x 10^{-7}M	solvent alone
r/r	16.3	28.9
r/o	17.9	30.3
r/+	19.8	30.7
+/+	11.7	30.2

*Determined from data of Figure 6 at 5 x 10^{-5} M cryptopleurine concentration.

absence of the drug indicating that all populations of ribosomes tested were equally active. In the presence of cryptopleurine, ribosomes from the diploid strains possessing at least one r allele (r/r, r/o and r/+) were inhibited approximately 40% while ribosomes from the sensitive diploid strain was inhibited by approximately 60%.

Gel electrophoresis of ^{3}H-polyphenylalanine. Electrophoresis experiments were performed again to determine if ribosomes from resistant strains (r/r and r/o) synthesized longer polyphenylalanine peptides than ribosomes from the sensitive strain (+/+) in the presence of cryptopleurine. The products of ribosomes from the heterozygous strain (r/+) also were examined to determine if they synthesized polyphenylalanine of intermediate size.

To examine the size of the ^{3}H-polyphenylalanine synthesized in vitro in the presence of 1×10^{-4} M cryptopleurine, electrophoresis samples were prepared as described above. The molecular weight distributions of ^{3}H-polyphenylalanine are illustrated in Figure 7. The patterns observed for ^{3}H-polyphenylalanine synthesized by ribosomes from each of the strains possessing at least one r allele (r/r, r/+ and r/o) were of similar sizes, heterogeneous, and indicate high molecular weight polyphenylalanine (9000 to 65,000 daltons). Ribosomes from the sensitive strain (+/+) synthesized nothing longer than oligopeptides and considerably less polyphenylalanine. Thus the sensitive ribosomes were not blocking other ribosomes progressing along the polyuridylic acid.

Data from the experiments described above and data for growth of cryptopleurine mutants on gradient plates (Meade, Riley and Manney (16)) are summarized in Table 7. From the gradient plate results and the

Figure 7. Electrophoresis of ^{3}H-polyphenylalanine. Electrophoresis samples were prepared and analyzed as described in the text. Protein standards are described in Fig. 5 with an additional standard, insulin (I), 5,700 daltons. The total number of ^{3}H-cpm incorporated into polyphenylalanine were for +/+, $1.38_4 \times 10^4$ (●); for r/r, 4.42×10^4 (■); for r/+, 4.86×10^4 (▲); and for r/o, 3.73×10^4 (●).

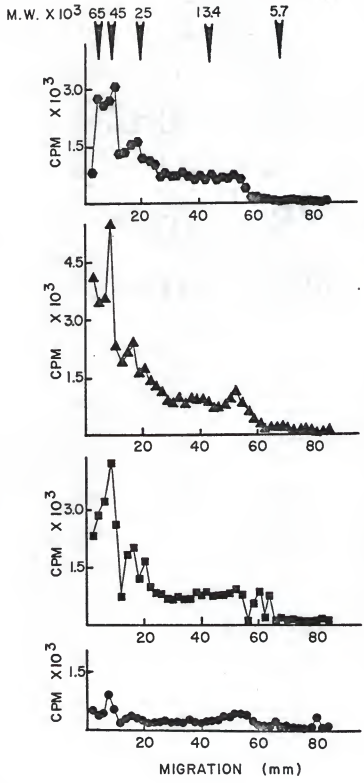

M.W. X 10³ 65 45 25 13.4 5.7
CPM X 10³
3.0
1.5
4.5
20
40
60
80
MIGRATION (mm)

Table 7. Summary of results

Strain	Cryl-7 allele[a] / ploidy	CRY resistance on gradient plates[b]	Ribosome yield ($\frac{\text{mg rib.}}{\text{gm cells}}$)[c]	Polyphenylalanine synthesis (CRY conc. ID_{50} μM)[d]	Protein synthesis in spheroplasts (CRY conc. ID_{50} μM)[d]	Polyphenylalanine produced/ribosome ($\frac{pM\ ^3H\text{-Phe}}{A_{260}}$ rib./15 min.)[e]
+	0	0	---	---	0.054	---
+/+	0	0	8.6	3.3	0.075	11.7
r/+	½	2	6.8	6.5	0.28	19.8
r/o	½	1	5.9	6.5	0.165	17.9
r/r	1	9	7.8	6.5	9.2	16.3
r	1	9	---	---	7.8	---

[a] From Table 4.

[b] From Meade, Riley and Manney (16). Scale increases from 0-9 with increasing cryptopleurine concentration.

[c] From Table 5.

[d] From Figure 6.

[e] From Table 6.

results of protein synthesis in spheroplasts, it is apparent that resistance to cryptopleurine *in vivo* depends on the ratio of *cryl* alleles to ploidy. The results of *in vitro* polyphenylalanine synthesis tests indicate that the presence of at least one r allele confers resistance to the ribosomes in these assays. The gel electrophoresis data of protein synthesized *in vivo* by spheroplasts and polyphenylalanine synthesized *in vitro* show that the three strains possessing at least one resistant allele synthesize similar amounts and sizes of protein. The sensitive strain synthesizes significantly less protein than the other strains, but it synthesizes the same size protein as the strains possessing one or more resistance allele. These results seem inconsistant and warrent explanation by a molecular model of cryptopleurine resistance in diploid yeast strains.

DISCUSSION

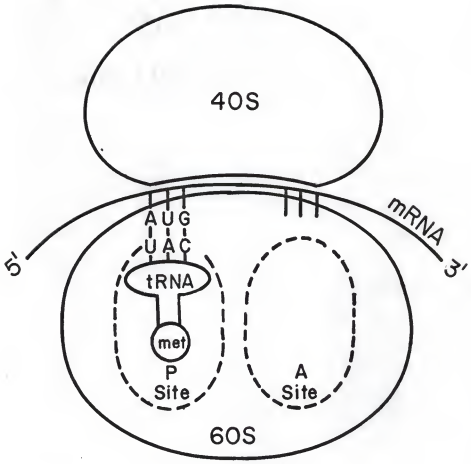
The object of this thesis was to further investigate the effects of gene dosage on the inhibition of protein synthesis by cryptopleurine in the yeast Saccharomyces cerevisiae. Resistance to cryptopleurine in diploid and higher ploidy yeast strains has been shown to depend not on the recessive or dominate character of the cryl gene but on the ratio of cryl alleles to ploidy (16). This unique form of genetic control was especially obvious in the monosomic diploid strains (r/o) which possessed only intermediate resistance to cryptopleurine on gradient agar plates. Protein synthesis was studied to quantitate the rates of protein synthesis in vivo in spheroplasts and to determine the resistance to cryptopleurine of ribosomes from several strains by in vitro polyphenylalanine synthesis. In order to understand the genetic control of cryptopleurine inhibition of protein synthesis in yeast, it is necessary to understand the transfer of information from the gene to the polypeptide and the basic steps of protein biosynthesis.

The genetic continuity of living organisms consists of three basic parts: 1) replication of the genetic material deoxyribonucleic acid (DNA), 2) transcription of the genetic information in DNA to the messenger ribonucleic acid (mRNA), 3) and translation of the mRNA into protein. Replication of the DNA is accomplished by a precise unfolding of the template DNA helix and duplication of its nucleic acid base chains. Transcription is accomplished when the template DNA unfolds and each DNA base is paired with its complimentary RNA base forming an mRNA that carries the genetic information of the DNA. The genetic code is triplicate

and therefore three specific RNA bases form a codon for each of the twenty amino acids used to produce the proteins. A given protein consists of a unique linear sequence of amino acids. The ribosome is the site of protein synthesis where the mRNA is "read" one triplet codon at a time, and the transfer ribonucleic acid (tRNA) with its matching anticodon delivers the specified amino acids necessary to synthesize protein. For example, the artificial mRNA polyuridylic acid (poly U) is translated by ribosomes into polyphenylalanine, indicating that the triplicate mRNA codon for the amino acid phenylalanine is UUU.

Eucaryotic ribosomes have a sedimentation coefficient of 80S and consist of two subunits, a large 60S subunit and a smaller 40S subunit. Protein synthesis begins with the formation of an initiation complex. The 40S subunit binds to the initiator $tRNA_{met_f}$ through the action of the initiation factor 2 (IF2) (27) and the hydrolysis of guanosine triphosphate (GTP). The mRNA is attached through the action of the initiation factor 3 (IF3) and the initiation factor 1 (IF1), although ill-defined, is thought to stabilize the initiation complex. The initiation complex then associates with the 60S subunit to form the intact ribosome. The large subunit possesses two sites, the peptidyl site (P site) and the aminoacyl site (A site) (see Figure 8). When the two subunits associate, the initiator, methionyl-tRNA, binds to the P site and the mRNA is positioned such that initiator codon is aligned with the met-tRNA anticodon. Next the aminoacylated tRNA specified in the mRNA binds to the A site positioning its anticodon with the next mRNA codon. This reaction is dependent on elongation factor 1 (EF1) and GTP. At this point, the methionine group bound to the tRNA at the P site

Figure 8. General model of a ribosome.


40S
mRNA
5'
3'
A U G
U A C
tRNA
met
P
Site
A
Site
60S

leaves the tRNA and forms a peptide bond with the amino acid attached to the tRNA at the A site. This peptidyl transfer reaction is catalyzed by the enyzme peptidyl transferase, which is an integral part of the 60S ribosomal subunit. Translocation is the next step in protein synthesis and involves two simultaneous events, the di-peptidyl-tRNA shifts from the A site to the P site dislodging the tRNA and the ribosome moves to the next codon of the mRNA. This step depends upon the presence of elongation factor 2 (EF2) and GTP. Elongation then procedes in a cyclic process. When the ribosome has proceded sufficiently along the mRNA, other ribosomes can attach, forming a polysome. Protein synthesis is terminated when the ribosome reaches a termination codon on the mRNA and this reaction requires several termination factors.

Most studies of protein synthesis in vitro use synthetic polymers such as polyuridylic acid as mRNA which lacks an initiator codon. High (Mg^{++}) are required for efficient translation of these messages and there is no requirement for the initiation factors required for protein synthesis in vivo. The ribosomes attach to the poly U and procede to elongate the polypeptide as described above. In yeast, however, in addition to EF1 and EF2, there is an elongation factor 3 (EF3) that is a ribosome-dependent GTPase and is required for polyphenylalanine synthesis (26). The ribosomes continue to the end of the poly U, release the polyphenylalanine, and dissociate into their component subunits.

Polyphenylalanine synthesis is inhibited by cryptopleurine by prevention of ^{3}H-Phe incorporation into polypeptide (2, 4, 7, 8, 10, 22, 25). The drug also has been described as inhibiting the elongation of nascent polypeptide as measured by amino acid incorporation (1, 8, 12, 22).

Through puromycin reaction assays the peptidyl transfer step was implicated to be the site of inhibition by one investigator (1) at very high concentration while other investigators have indicated that translocation is the site of inhibition (1, 4, 8, 12, 22). Further evidence that translocation is the step inhibited by cryptopleurine is that Barbacid, Fresno and Vazquez (1) observed that cryptopleurine inhibited the step in translocation which requires EF2, and Bucher and Skogerson (4) found that EF2 and GTP competitively inhibit cryptopleurine's effect on translocation.

Although it is believed that cryptopleurine inhibits translocation, no specific binding site for the drug has been identified. Cryptopleurine binds to yeast 80S ribosomes (2, 4, 10) and to isolated 40S and 60S yeast ribosomal subunits, but since it binds to ribosomes in an amount proportional to the cryptopleurine concentration, there does not seem to be a saturable ribosomal binding site for the drug (4). One investigator observed that cryptopleurine acted specifically on the yeast 60S subunit when tested at very high concentrations (2) while others (4, 7, 8, 22, 25) reported that the 40S subunit was the site of cryptopleurine resistance in mutant strains of yeast. Investigators also found 40S subunits from the mutant cryptopleurine resistant strains to be less stable than those from cryptopleurine sensitive strains (4, 7, 8, 25). Buchner and Skogerson (4) suggested that cryptopleurine either binds to the 40S subunit or it effects the association of the 40S and 60S subunits since the 40S subunit is the site of cryptopleurine resistance and the 60S subunit is the site of translocation.

The experiments reported in this thesis were performed to investigate the effects of gene dosage on cryptopleurine inhibition of protein synthesis in diploid strains of the yeast Saccharomyces cerevisiae. The protein synthesis studies in spheroplasts and in vitro with ribosomes were conducted to determine the sensitivities to cryptopleurine of the various mutants. Protein synthesized by spheroplasts and polyphenylalanine synthesized with ribosomes were analyzed by gel electrophoresis to determine if the protein synthesized by the various strains in the presence of cryptopleurine varied in size. From the data in Table 7, it is apparent that there are two types of resistance to cryptopleurine in these mutants: 1) in in vitro polyphenylalanine synthesis ribosomes from strains that possess at least one resistance allele were indistinguishable by this assay and were more resistant to cryptopleurine than were sensitive ribosomes, and 2) growth on gradient agar plates and protein synthesis in spheroplasts, in vivo resistance to cryptopleurine depended on the ratio of cryl alleles/ploidy. An explanation for the genetic control of cryptopleurine resistance must account for both the differences in the in vivo and in vitro assays and the results obtained using these assays. From the data, several molecular models for the basis of cryptopleurine resistance seem unlikely and therefore a model is discussed that suggests the existence of another locus, not on chromosome III, that regulates cryptopleurine resistance.

It has been suggested (16) that the cryl locus might code for a component of the 40S ribosomal subunit that causes the mutant ribosome to be resistant to cryptopleurine. This hypothesis predicts that yeast strains possessing only r alleles (r/r, r/o and r) would produce resistant

ribosomes with altered 40S ribosomal subunits and would be resistant to the drug. It also predicts that sensitive strains would produce normal ribosomes with unaltered 40S ribosomal subunits and they would be sensitive to cryptopleurine. The heterozygous diploid (r/+) would be expected to produce either a mixture of sensitive and resistant ribosomes, or hybrid ribosomes. If the r/+ strain produced a mixture of ribosomes it would possess an intermediate resistance that would depend on the percentage of resistant ribosomes. If the heterozygote (r/+) produced hybrid ribosomes, it would possess an intermediate resistance that would depend on the inherent resistance of the hybrid ribosomes. The hypothesis that a structural alteration of the 40S subunit causes a strain to be resistant to the drug does not account for these experiments in a straight forward manner since the heterozygous diploid (r/+) produced ribosomes that were fully resistant to cryptopleurine in cell-free synthesis of polyphenylalanine (Table 7). The data from protein synthesis in spheroplasts (Table 7) also conflicts with the above hypothesis since the monosomic diploid strain (r/o) was more sensitive to cryptopleurine than was the homozygous diploid strain (r/r). From these results, the basis for cryptopleurine resistance appears not to involve an alteration in the structure of the ribosome alone.

If cryptopleurine resistance in yeast is not caused by a structural alteration in the ribosome, it might be affected by a variation in resistant ribosome concentrations in cells of the same ploidy. All the diploid cells should have the same ribosome concentration. The monosomic diploid (r/o) might have possessed fewer ribosomes than the

fully diploid strains (r/r, r/+ and +/+). If this were the case, then the level of resistance exhibited by strains might have been proportional to the number of resistant ribosomes per cell. The r/+ and r/o strains might have been expected to display equal levels of resistance, since they could contain the same concentration of resistant ribosomes, although the r/+ strain might possess a greater number of ribosomes *in toto*. Meade, Riley and Manney (16) have shown that ratios of mean cell volume to ploidy in all the diploid strains tested were comparable and therefore that the strains contained the same number of cells per gram wet weight. As shown in Table 7, the amount of ribosomes per gram of cells (and therefore per given number of cells) was only slightly less for the monosomic diploid (r/o) than for the other strains. But as was mentioned before, the r/o strain produced 76% as many ribosomes as did the r/r strain and 87% as many as did the r/+ strain. Since the r/o strain should produce only resistant ribosomes, spheroplasts prepared from the r/o strain should be 76% as resistant as spheroplasts prepared from the r/r strain. The results from the test of protein synthesis in spheroplasts showed the r/o strain to be much less resistant than the r/r strain and therefore different concentrations of resistant ribosomes per cell alone can not account for the monosomic diploid's (r/o) lower resistance to cryptopleurine.

Since the various resistances to cryptopleurine are difficult to explain simply by an alteration of ribosomal structure or by the concentration of resistant ribosomes per cell, resistance may be affected by an interaction between the *cryl* gene product and the products of one or more other loci not on chromosome III. Two types of genetic control

that involve multiple gene loci are complimentary and polymeric control. In complementary gene control, there are several loci, each that codes for a different enzyme required for synthesis of an amino acid. A mutation at any one of these loci causes the strain to be auxotrophic for that amino acid, but two strains deficient for different enzymes can mate and complement the other strain's deficiency.

In polymeric control there are several unlinked genes that produce identical or nearly identical products. If one of the loci is deficient, the other loci may compensate by producing more product. A deficiency in one of the loci may also interfere with or affect the product of the other loci. Meade, Riley and Manney (16) reported a cryptopleurine resistant mutant that appeared to require two genes for the expression of its mutant phenotype. Neither of these genes appeared to be linked to MAT1 and to cry1, both on chromosome III. The existence of more than one gene that regulates a given phenotype is feasible, since Saccharomyces cerevisiae possesses 4 unlinked genes that control maltose fermentation and 5 to 7 unlinked genes for the production of each tRNA (6).

In order to understand the differing results obtained in in vivo protein synthesis in spheroplasts and in in vitro polyphenylalanine synthesis, the differences between the two types of assays must be considered. Polyphenylalanine synthesis used high (Mg^{++}) and a poly U message. This assay did not initiate or terminate in the normal fashion and was therefore a measure of elongation. The elongation factors in this assay were obtained from the sensitive diploid and thus resistance to cryptopleurine can be attributed to the ribosomes.

Protein synthesis in vivo in spheroplasts used nascent mRNA and since the (Mg++) was naturally low the message was initiated, elongated, and terminated through the natural sequence. Cryptopleurine resistance in spheroplasts can not be attributed rigorously to the ribosomes since the elongation factors used were the ones inherent to each strain. Previous investigators found (8, 25) that elongation factors were not involved in cryptopleurine resistance in haploids when measured by polyphenylalanine synthesis, but it is not known whether elongation factors influence cryptopleurine resistance in vivo.

A model consistant with the data presented is that a gene on a chromosome other than chromosome III, denoted crym for cryptopleurine modifier, produces a component of the P site on the 60S ribosomal subunit or one of the initiation factors. The cryl locus produces 40S ribosomal subunits that are more resistant to cryptopleurine. The presence of a sensitive allele (+) at the cryl locus therefore, is not the presence of sensitive 40S subunits coded by that locus, but the lack of resistant subunits. Other loci then code for both normal ribosomal subunits. During initiation of protein synthesis there is possibly a competition between the resistant 40S ribosomal subunits modified by the cryl gene product and the normal 40S subunits for the 60S subunits or the initiation factors produced by the crym locus.

This model predicts that in in vitro polyphenylalanine synthesis experiments, where elongation is the only protein synthesis step measured, the ribosomes from the strains with at least one r allele would be more resistant to cryptopleurine. In the tests of poly U directed polyphenylalanine synthesis (Figure 6) the ribosomes from the

strains with at least one r allele possessed equal resistance and were more resistant than ribosomes from the sensitive strain.

In in vivo protein synthesis experiments initiation and elongation are both measured by the incorporation of radioactive amino acids. Resistance to cryptopleurine could be a mixture of resistance due to the resistant 40S subunit in the elongation step and interaction of the resistant subunit with 60S subunits or initiation factors in the initiation step of protein synthesis. The resistant 40S subunit could interact with either an initiation factor or the P site on the 60S subunit, where the initiator tRNA is initially placed, and cause the complex to be more resistant to cryptopleurine during initiation. While the strains that contain at least one r allele produce resistant 40S subunits and elongate protein in the presence of cryptopleurine these subunits are also important in causing resistance to cryptopleurine during initiation. The r/+ and r/o strains, with a ratio of cryl alleles/ploidy of 0.5, have fewer resistant ribosomes to complex with the initiation factors or 60S subunits than the r/r or r strains and are therefore less resistant. The resistance to cryptopleurine in initiation could depend on the ratio of cryptopleurine resistance ribosomes to the amount of initiation factors or 60S subunits.

The crym model also predicts that in vitro the strains possessing a cryl allele and therefore resistant ribosomes should produce the same size and more peptide than ribosomes from the sensitive strain. This is supported by Figure 7 where the proteins synthesized by the ribosomes in the presence of cryptopleurine were of the same size, but the sensitive strain produced considerably less protein. This model predicts that

in vivo the strains possessing an r allele would elongate protein better and produce more protein, but that the r/r strain should initiate better and produce more peptide than the r/+ and r/o strains. In Figure 5, the strains with one r allele did produce the same size and more protein, but the yields for the r/r, r/+ and r/o strains were comparable. This may be due to the excess ^{3}H-Leu added and the longer pulse time used to produce proteins for gel electrophoresis. The resistance to cryptopleurine is probably produced by a complex balance of initiation and elongation resistances and is affected by experimental conditions.

Previous investigators (8, 11, 24) had ruled out initiation as a site of cryptopleurine action since polysome sucrose gradient profiles from sensitive strains did not change in the presence of cryptopleurine. When a strain is inhibited at the initiation step, its polysomes profile degrades to single ribosomes. But if elongation and initiation were affected, the ribosomes would be "frozen" on the message and the defect in initiation would not be distinguishable. Cryptopleurine therefore might affect initiation as well as elongation measured in these tests.

These results indicate that cryptopleurine resistance in yeast may be controlled by more than one genetic locus. Experiments to isolate and investigate alternate cryptopleurine mutants at the *crym* locus are necessary for further development of a molecular model of cryptopleurine resistance. It would also be useful to know if haploid strains disomic for chromosome III, and therefore possessing a *cryl*/ploidy ratio of 2, are more resistant than strains possessing a ratio of 1. Cryptopleurine resistance in yeast continues to provide an interesting system for

analyzing some effects of gene dosage on the inhibition of protein synthesis in yeast.

APPENDIX I

Nomenclature For Genetic Markers

Nutritional Requirement	Symbol
Adenine	ade
Leucine	leu
Lysine	lys
Methionine	met
Histidine	his
Threonine	thr
Tryptophan	trp
Uracil	ura

Others:

Cryptopleurine (resistance)	cry
Galactose (loss of fermentation ability)	gal
Mating Type	$\underline{a}$, $\underline{\alpha}$

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THE INFLUENCE OF GENE DOSAGE ON THE INHIBITION OF PROTEIN SYNTHESIS BY CRYPTOPLEURINE IN THE YEAST SACCHAROMYCES CEREVISIAE

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Inhibition of protein synthesis by cryptopleurine was studied in spheroplasts from the yeast Saccharomyces cerevisiae and with purified yeast ribosomes in polyuridylic acid directed polyphenylalanine synthesis in order to better understand the genetic regulation of cryptopleurine resistance. A monosomic diploid strain possessing one cryptopleurine resistance allele was of special interest since it was not resistant to crytopleurine on agar plates containing a gradient of cryptopleurine concentrations.

Rates of protein synthesis were quantitated in spheroplasts from several strains of yeast, including the monosomic diploid. The yeast strains expressed various resistances to cryptopleurine such that strains with a higher ratio of cryl-7 alleles to ploidy were more resistant than strains with lower ratios. Strains with similar ratios displayed similar levels of resistance to the drug.

Cryptopleurine inhibition of polyphenylalanine synthesis was studied in vitro using soluble elongation factors prepared from the sensitive diploid strain. The ribosomes used in the study were prepared from the sensitive diploid strain, a resistant diploid strain, a heterozygous diploid strain, and a monosomic diploid strain. Ribosomes obtained from all strains possessing at least one resistant allele were indistinguishable from each other by this assay and were more resistant to cryptopleurine than were ribosomes from the sensitive strain.

A model is discussed that suggests the existence of a locus not on chromosome III whose product interacts with the cryl gene product to affect the expression of cryptopleurine resistance in yeast.